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ORIGINAL ARTICLE

MicroRNA-15a-5p suppresses cancer proliferation and division in human hepatocellular carcinoma by targeting BDNF

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Abstract We examined the expression pattern and functional roles of microRNA 15a-5p (miR-15a-5p) in human hepatocellular carcinoma (HCC). Possible miR-15a-5p aberrant expression in HCC cell lines or clinical HCC specimens was examined by quantitative real-time PCR (qRT-PCR). In HCC HepG2 and SNU-182 cells, miR-15a-5p was ectopically overexpressed by lentiviral transduction. Its effect on HCC proliferation, cancer division, and in vivo tumor growth were examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cell cycle assay, and tumorigenicity assay, respectively. The targeting of miR-15a-5p on its downstream gene, brain-derived neurotrophic factor (BDNF), was examined by dual-luciferase assay, qRT-PCR, and Western blot, respectively. BDNF was then overexpressed in HepG2 and SNU-182 cells to evaluate its selective effect on miR-15a-5p in HCC modulation. MiR-15a-5p is aberrantly downregulated in in vitro HCC cell lines and in vivo HCC clinical specimens. Ectopic overexpression of miR-15a-5p suppressed cancer proliferation, induced cell cycle arrest in HepG2 or SNU-182 cells in vitro, and inhibited HCC tumor growth in vivo. MiR-15a-5p selectively and negatively regulated BDNF at both gene and protein levels in HCC cells. Forced overexpression of BDNF effectively reversed the tumor suppressive functions of miR-15a-5p on HCC proliferation and cell division in vitro. Our study demonstrated that miR-15a-5p is a tumor suppressor in HCC and its regulation is through BDNF in HCC.

Keywords Hepatocellular carcinoma · miR-15a-5p · BDNF · Cancer proliferation · Cell cycle

Introduction

Hepatocellular carcinoma (HCC) is one of the most malign cancers for both men and women, representing the third most common cancer-related deaths in the world [1]. In 2015, the estimated HCC-related new cancer cases exceed 35,000 and the estimated HCC-related cancer deaths are more than 24,000 in the USA [2]. In the last few decades, medical investigators had made great strides in making better evidence-based diagnosis and providing optimal treatment plans for patients with HCC [3]. In addition, many biomarkers and targeted genes have been identified for early diagnosis of HCC [4]. However, given the fact that the overall prognosis or 5-year survival rates are still poor in patients with HCC, especially the patients in eastern Asian countries including China [1], continuous effort is much needed to further explore the underlying mechanisms of HCC to deliver better treatment strategies for patients with HCC.

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MicroRNAs (miRNAs) are groups of evolutionarily conserved, small-length noncoding RNAs that post-transcriptionally suppress gene expression through the direct binding with the 3′-untranslated region (3′-UTR) of target genes [5]. In human cancers, miRNAs may act as either oncogenic factors or tumor suppressors, based on the regulated tumor forms and their targeted genes [6]. In human HCC, miR-106b-25 cluster was found to play oncogenic role in HCC by suppressing the ectopic expression of apoptotic gene E2F1 [7]. On the other hand, several miRNAs, including miR-122 and miR-124, were found to be downregulated in HCC, thus having tumor suppressive roles in regulating HCC [8–10]. Among many of the tumor suppressing miRNAs, member of miR-15a family, miR-15a-3p, was found to inhibit cancer proliferation and induce cancer apoptosis in prostate cancer and various human cancer cell lines [11, 12]. Interestingly, little is known for the expression pattern or functional roles of the other member of mature miR-15a family, miR-15a-5p, in human cancers, or HCC.

Brain-derived neurotrophic factor (BDNF) is a pro-neuronal transcriptional factor, playing important role in neural maturation and differentiation [13]. It was recently discovered that BDNF was also a critical cancer regulator [14–16]. In HCC, it was found that BDNF was highly expressed in HCC cell lines and HCC tumors, and the application of BDNF neutralizing antibody inhibited proliferation and induced apoptosis in HepG2 and HCCLM3 cells [16]. However, little is known for the upstream molecular pathways of BDNF in HCC.

In this work, we firstly discovered that miR-15a-5p was aberrantly downregulated in both HCC cell lines and clinical HCC specimens. We then demonstrated that ectopic overexpression of miR-15a-5p exerted tumor suppressive effect on HCC. Furthermore, we utilized molecular biochemistry and bioinformatic method to demonstrate that pro-oncogenic gene BDNF was actively involved in the regulation of miR-15a-5p in HCC.

Materials and methods

Ethic statement

In the present study, all participating patients signed consent forms. All clinical procedures were approved by the Ethic Committee at The First Affiliated Hospital, Sun Yat-sen University in Guangzhou, Guangdong Province, China. All experiments were conducted in accordance with the Declaration of Helsinki and the federal regulation on human research in the People's Republic of China.

HCC cell culture and clinical specimens

There were eight human HCC cell lines included in the study. Among them, HepG2, Huh7, SNU-398, SNU-182, SNU-449, and SK-Hep1 were obtained from the American Type Culture Collection (ATCC, USA). The other two cell lines, HuH7 and MHCC97L, were obtained from the China Center for Type Culture Collection (CCTCC, Wuhan, China). Two immortal normal hepatic cell lines, MIHA and THLE2, were obtained from ATCC. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM-high-glucose, Invitrogen, USA) supplemented with 10 % fetal calf serum (Invitrogen, USA) in a tissue culture incubator at 37 °C with 5 % CO_2.

Clinical specimens of liver tumors (T) were collected from 13 HCC patients who had hepatic surgery. The adjacent non-tumor tissues (ANT) were collected at least 4 cm from the clear edge of tumor. After extraction, all specimens were immediately snap-frozen in liquid nitrogen and stored at −80 °C until further use.

RNA extraction and quantitative real-time reverse transcription-PCR

Total RNA was extracted from HCC cell lines or clinical specimens using TRIzol reagent (Invitrogen, USA), and then reverse transcribed into complementary DNA (cDNA) with SuperScript II reverse transcriptase enzyme (Invitrogen, USA). Gene expression of miR-15a-5p was examined by quantitative real-time reverse transcription-PCR (qRT-PCR) with a TaqMan MicroRNA Assays (Applied Biosystems, USA) and internal control of U6 RNA. Gene expression of BDNF was examined with a SYBR Green Real-Time PCR Master Mix Kit (Applied Biosystems, USA) and internal control of 18 s gene. All qRT-PCR reactions were carried out on an ABI PRISM ® 7700 Sequence Detection System (Applied Biosystems, USA) according to the manufacturer's instructions. Relative gene expression was evaluated as folder changes using $2^{-\Delta\Delta Ct}$ method.

Lentivirus production

Lentivirus of human mature miR-15a-5p mimics (Lenti-miR15a-5p), as well as a negative control miRNA lentivirus (Lenti-miRC), was obtained from SunBio (SunBio Medical Biotechnology, China). HCC cell lines, HepG2 and SNU-182, were transduced with Lenti-miR15a-5p or Lenti-miRC (10^8 particles/mL) with a multiplicity of infection (MOI) of 50 and 6 μg/mL polybrene for 24 h. HCC cells were then maintained in 6-well plates and purified with 5 μg/mL puromycin for 1 week before further experiments.

HCC proliferation

The proliferation rates of HepG2 and SNU-182 cells were monitored using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Invitrogen, USA) according to the manufacturer's instructions. In 96-well plate, 5000 (per well) lentivirus-transduced HepG2 and SNU-182 cells

were maintained for 5 days. Every 24 h, the culture was introduced with 15 μL 5 mg/mL MTT solution (Invitrogen, USA) for 4 h, followed by three times wash of PBS and 15-min agitation of 0.15 mL dimethyl sulfoxide solution (DMSO, Invitrogen, USA). The 96-well plates were then placed into a SpectraMax M5 fluorescence microplate reader (Molecular Devices, USA). The relative proliferation rates were examined at absorbance of 570 nm according to the manufacturer's instructions.

HCC cell cycle

HepG2 and SNU-182 cells were fixed with ice-cold 70 % ethanol, and then stained with propidium iodide (50 μg/mL, BD Biosciences, USA). Cells were sorted by FACSCalibur™ flow cytometry (BD Biosciences, USA), and the percentages of cells in G0/G1, S, and G2/M stages were calculated by a Multicycle AV software (Phoenix; Phoenix Flow System, USA) according to the manufacturer's instructions.

HCC tumorigenicity

Stably lentiviral transduced HepG2 cells (1×10^7 cells) were subcutaneously inoculated into the left flanks of female athymic mice (6-week-old). The in vivo tumor sizes were measured every week for 5 weeks, and the tumor volumes (mm^3) were calculated as length × $width^2$ / 2. Five weeks after in vivo tumorigenicity assay, mice were sacrificed and tumors were retrieved for imaging.

Dual-luciferase reporter assay

The hsa-miR-15a-5p targeting sequence at the wild type (wt) 3′-untranslated region (3′-UTR) of human BDNF (NCBI RefSeq ID: NM_170731) was amplified from a human cDNA library. After verified by sequencing, the cDNA sequence was cloned into a pmiR-REPORT luciferase plasmid (Applied Biosystems, USA) downstream of the HindIII restriction site (wtBDNF). The sequence was also mutated by a QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, USA) to nullify the binding of hsa-miR-15a-5p, and then cloned into pmiR-REPORT (mtBDNF). An empty pmiR-REPORT was used as control. Human HEK293T cells were co-infected with miR-15a-5p mimics, and wtBDNF, mtBDNF, or control luciferase plasmid for four times (1 h each time with 1-h interval). Forty-eight hours after infection, cells were harvested and relative firefly luciferase activities were measured with a dual-

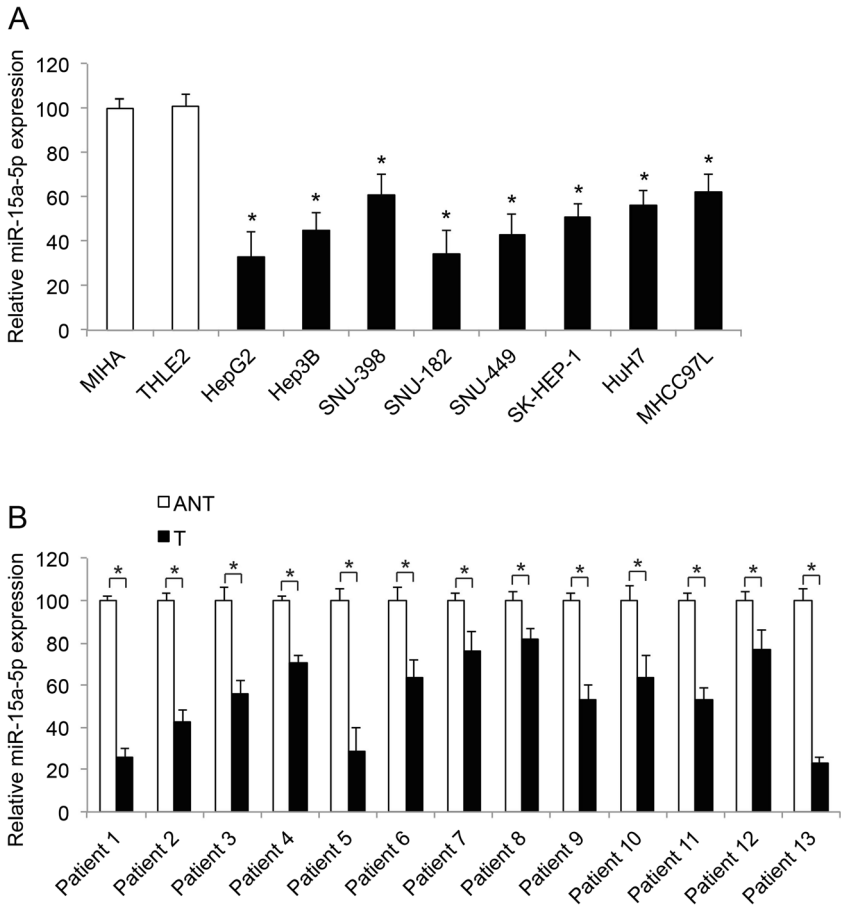

Fig. 1 MiR-15a-5p is downregulated in HCC. **a** QRT-PCR was used to measure the miR-15a-5p expression in eight HCC cell lines, HepG2, HepG3B, SNU-398, SNU-182, SNU-449, SK-HEP-1, HuH7, and MHCC97L cells, against two non-tumorous immortal liver cell lines, MIHA and THLE2 cells. (*$P<0.05$). **b** QRT-PCR analysis of miR-15a-5p expression between tumor (*T*) and adjacent non-tumor (*ANT*) liver specimens in 13 cohort patients with HCC (*$P<0.05$)

luciferase reporter assay (Promega, USA) according to the manufacturer's instructions.

Western blot analysis

HepG2 and SNU-182 cell cultures were washed with PBS and lysed with ice-cold RIPA buffer (150 mM NaCl with 50 mM, pH=7.8, Roche, USA). Equal proteins (40 μg/sample) were fractionated on a 10 % SDS–PAGE gel and then transferred to a PVDF membrane. The membrane was treated by a primary antibody against human BDNF (Sigma-Aldrich, USA) at 1:500 ratio over night at 4 °C, followed by a horseradish peroxidase conjugated secondary antibody 1:1000 ratio at 37 °C for 2 h. The blot was developed with ECL+ reagent (Amersham Biosciences, USA) and visualized with an enhanced chemiluminescence film system (Amersham Pharmacia Biotechnology, USA).

BDNF overexpression

Whole DNA sequence of human BDNF was copied from a human cDNA library and verified by sequencing. It was then cloned into the eukaryotic expression vector pcDNA3.1 to make the BDNF overexpression plasmid, pcDNA3.1/BDNF. HepG2 and SNU-182 cells were transfected with pcDNA3.1/BDNF to induce ectopic BDNF overexpression in HCC cells. The control HCC cells were transfected with an empty pcDNA3.1 vector, pcDNA3.1/+. The efficiency of BDNF overexpression assay was evaluated by qRT-PCR.

Statistical analysis

All data were presented as mean value±standard errors. The independent two-tails unpaired Student's *t* test was used for statistical comparisons across the study. A *P* value less than 0.05 was considered statistically significant.

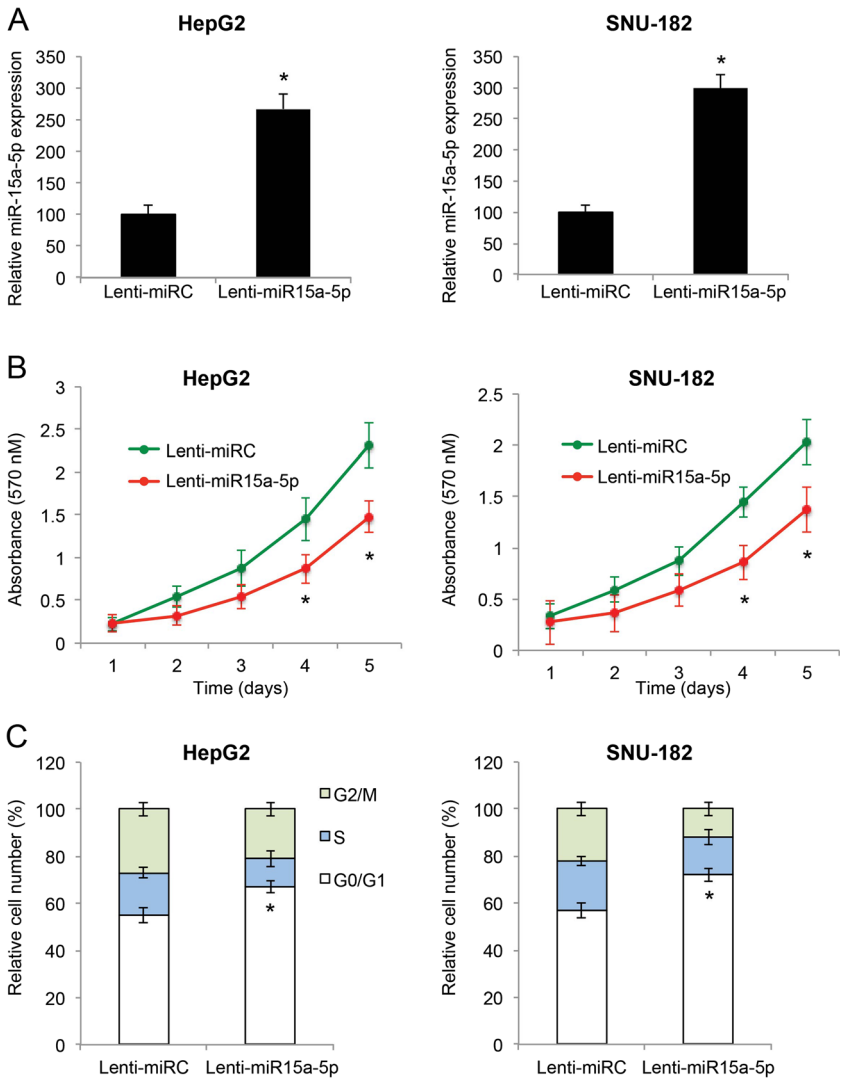

Fig. 2 MiR-15a-5p overexpression suppressed HCC proliferation and cell division. **a** HepG2 and SNU-182 cells were transduced with lentiviruses expressing miR-15a-5p mimics (Lenti-miR15a-5p), or negative control miRNA (Lenti-miRC). QRT-PCR confirmed that Lenti-miR15a-5p significantly upregulated miR-15a-5p expressions in both HepG2 and SNU-182 cells (*P<0.05). **b** An in vitro MTT assay was carried out for 5 days to evaluate cancer cell proliferation in HepG2 and SNU-182 cells after lentiviral transduction (*P<0.05). **c** HepG2 and SNU-182 cells were fixed with ice-cold 70 % ethanol and stained with propidium iodide. FACS sorting was carried out to measure the percentages of HCC cells in G0/G1, S, or G2/M stages during cell division (*P<0.05)

Results

MiR-15a-5p is downregulated in both HCC cells and clinical specimens

In our work, we firstly examined whether miR-15a-5p was aberrantly expressed in HCC. We applied qRT-PCR technology and examined miR-15a-5p expression in eight HCC cell lines, HepG2, HepG3B, SNU-398, SNU-182, SNU-449, SK-HEP-1, HuH7, and MHCC97L cells, as well as two non-tumorous immortal liver cell lines, MIHA and THLE2 cells. The result showed that miR-15a-5p was aberrantly downregulated in all HCC cell lines, as compared to non-tumorous liver cell lines (Fig. 1a, $*P<0.05$). To validate whether aberrant downregulation of miR-15a-5p was also the case in clinical specimens, we then examined miR-15a-5p expression in 13 cohort HCC patients. The result confirmed that in each patient, miR-15a-5p was downregulated in HCC tumors (T), as compared to adjacent non-tumor (ANT) liver specimens (Fig. 1b, $*P<0.05$).

Overexpression of miR-15a-5p negatively regulated HCC proliferation and cell cycle in vitro

As we discovered that miR-15a-5p was aberrantly downregulated in HCC, we hypothesized that miR-15a-5p overexpression may functionally regulate HCC. Thus, we transduced two HCC cell lines, HepG2 and SNU-182 cells, with lentivirus expressing miR-15a-5p mimics (Lenti-miR15a-5p) to ectopically overexpress miR-15a-5p. HCC cells were also transduced with a lentivirus containing a negative control miRNA (Lenti-miRC). After lentiviral transduction was stabilized, the overexpression efficiency was confirmed by qRT-PCR (Fig. 2a, $*P<0.05$).

We then explored the effect of miR-15a-5p overexpression on HCC proliferation in vitro. MTT assay showed that in both HepG2 and SNU-182 cells, growth rates were significantly decreased by miR-15a-5p overexpression (Fig. 2b, $*P<0.05$). To better understand its inhibitory mechanism, we further examined the effect of miR-15a-5p overexpression on HCC cell cycle regulation. The result showed that miR-15a-5p overexpression suppressed HCC cell cycle as more HepG2 or SNU-182 cells were arrested at G0/G1 stage (Fig. 2c, $*P<0.05$).

Therefore, our results demonstrated that overexpression of miR-15a-5p negatively regulated HCC growth by inhibiting cancer proliferation and inducing cell cycle arrest.

Overexpression of miR-15a-5p suppressed HCC tumorigenicity in vivo

We then explored the effect of miR-15a-5p overexpression on in vivo growth of HCC tumors. After lentiviral transduction of miR-15a-5p mimics or control miRNA, HepG2 cells were subcutaneously injected into the left flanks of female athymic null mice (6-week-old). The weekly in vivo tumorigenicity assay showed that tumor volumes (mm^3), estimated as length × $width^2$ / 2, were significantly smaller in mice injected with miR-15a-5p overexpressed HepG2 cells, than in mice injected with control HepG2 cells (Fig. 3a, b, $*P<0.05$).

MiR-15a-5p regulates BDNF in HCC

We hypothesized that miR-15a-5p inhibited HCC proliferation and cell division through the regulation on its target genes. We searched several databases to seek the possible downstream target genes of miR-15a-5p, including miRANDA and TargetScan. We identified that a pro-tumor gene, brain-derived neurotrophic factor (BDNF), was very likely to be the candidate (Fig. 4a). In order to confirm the direct targeting of miR-15a-5p on BDNF gene, we carried out a dual-luciferase reporter assay. It showed that miR-15a-5p significantly decreased the luciferase activity of wild type BDNF

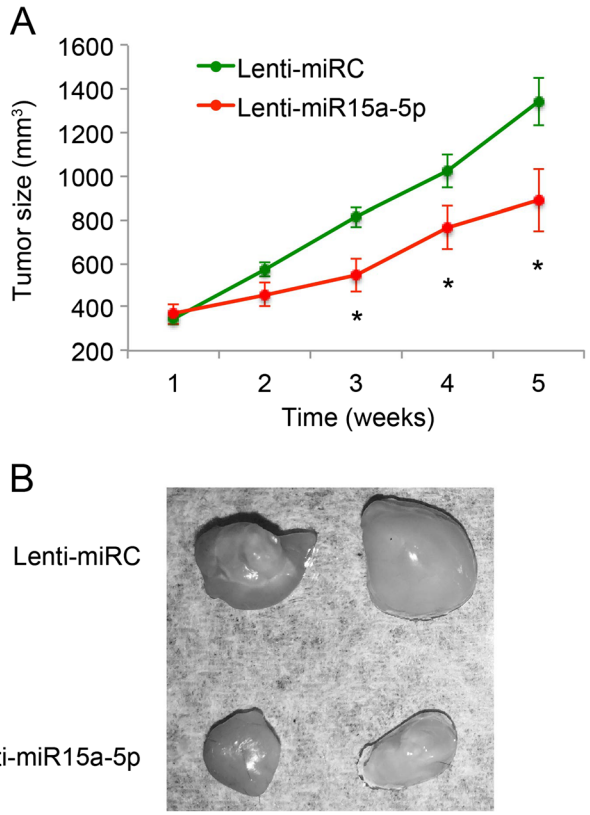

Fig. 3 MiR-15a-5p overexpression suppressed HCC tumorigenicity. **a** After lentiviral transduction, miR-15a-5p overexpressed HepG2 cells were subcutaneously inoculated into the left flank of a 6-week-old female athymic null mice (10^7 cells per injection). The control mice were injected with same amount of HepG2 cells which were transduced with Lenti-miRC. In vivo tumorigenicity was monitored weekly for 5 weeks. Tumor volumes were measured by length × $width^2$ /2 ($*P<0.05$). **b** At the end of in vivo tumorigenicity assay, mice were sacrificed and HCC tumors were extracted

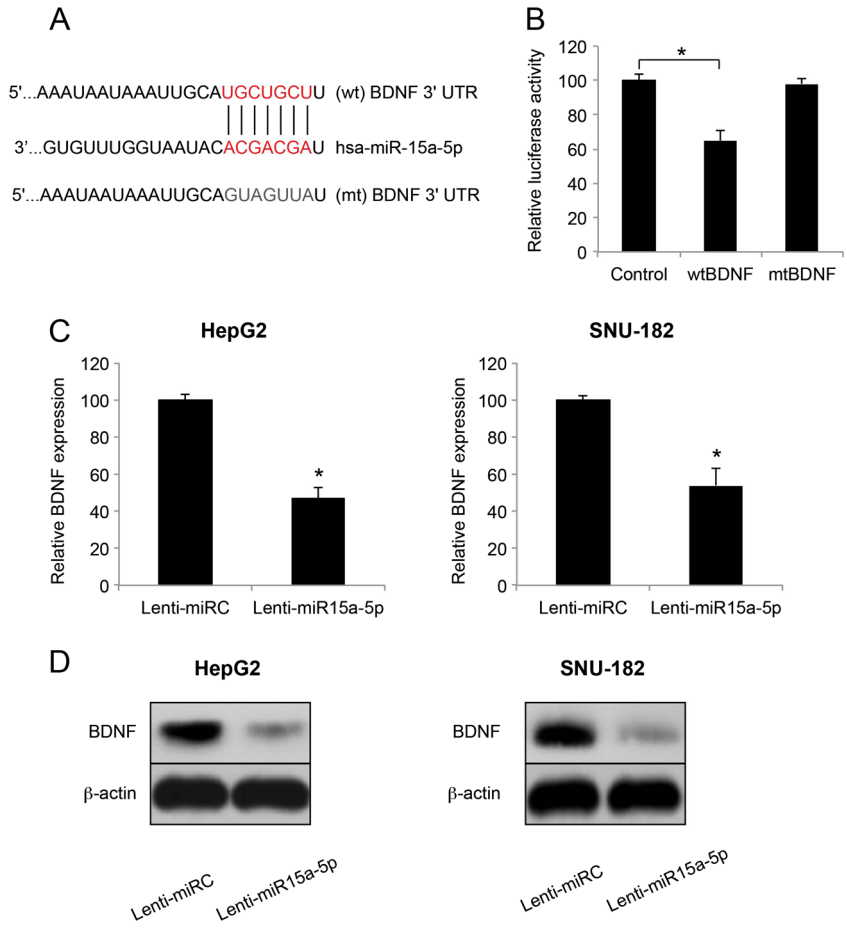

Fig. 4 MiR-15a-5p selectively targets BDNF in HCC. **a** The binding of has-miR-15a-5p on 3′-UTR of wild type (wt) BDNF is schematically shown. The mutated (mt) binding sequence of BDNF 3′-UTR was also presented. **b** HEK293T cells were co-infected with miR-15a-5p mimics, and one of the three luciferase plasmids, wtBDNF, mtBDNF, or control. Forty-eight hour after co-transfections, the relative luciferase activity of each plasmid was measured by a dual-luciferase reporter assay and normalized to control (*$P<0.05$). **c** After lentiviral transduction, gene expressions of BDNF were compared by qRT-PCR between miR-15a-5p overexpressed HepG2 or SNU-182 cells, and control cells (*$P<0.05$). **d** Protein expressions of BDNF were also compared between miR-15a-5p overexpressed HCC cells and control cells

(wtBDNF) reporter, but had little effect on the luciferase activity of mutant BDNF (mtBDNF) (Fig. 4b, *$P<0.05$). Thus, the dual-luciferase activity assay demonstrated that miR-15a-5p specifically regulated BDNF.

We then examined whether miR-15a-5p selectively modulated BDNF in HCC. In lentiviral transduced hepG2 and SNU-182 cells, we compared the gene expressions of BDNF between miR-15a-5p overexpressed HCC cells and control cells. The results of qRT-PCR showed that miR-15a-5p overexpression significantly suppressed BDNF gene expression in both HepG2 and SNU-182 cells (Fig. 4c, *$P<0.05$). Furthermore, we examined the protein levels of BDNF in HCC cells. The result of Western blot showed that miR-15a-5p overexpression also suppressed BDNF protein production in HepG2 and SNU-182 cells (Fig. 4d).

Overexpression of BDNF reversed the tumor suppressive effect of miR-15a-5p in HCC

We then hypothesized that BDNF could also modulate miR-15a-5p-related cancer regulation in HCC. To examine this hypothesis, we generated a BDNF overexpressing vector, pcDNA3.1/BDNF, and transfected it into miR-15a-5p overexpressed hepG2 and SNU-182 cells (transduced with Lenti-miR15a-5p). In control HCC cells, an empty pcDNA3.1/+ vector was used in transfection. The transfection efficiency was verified by qRT-PCR assay (Fig. 5a, *$P<0.05$) and Western blot assay (Fig. 5b). Then, we carried out a MTT assay to evaluate the effect of BDNF overexpression on cancer proliferation in miR-15a-5p overexpressed HepG2 and SNU-182 cells. It showed that BDNF overexpression increased the growth rates in both HepG2 and SNU-182 cells that were transduced with Lenti-miR15a-5p (Fig. 5c, *$P<0.05$). Furthermore, we examined the effect of BDNF overexpression on HCC cell division. It showed that BDNF overexpression restored HCC cell cycle transition from G0/G1 to S stage in both HepG2 and SNU-182 cells transduced with Lenti-miR15a-5p (Fig. 5d, *$P<0.05$).

Therefore, our data clearly demonstrated that overexpression of BDNF successfully reversed the tumor-suppressive effect of miR-15a-5p overexpression on cancer proliferation and cell division in HCC.

Discussions

In the last decade, epigenetic regulation of miRNAs has shown be to an important factor in modulating HCC

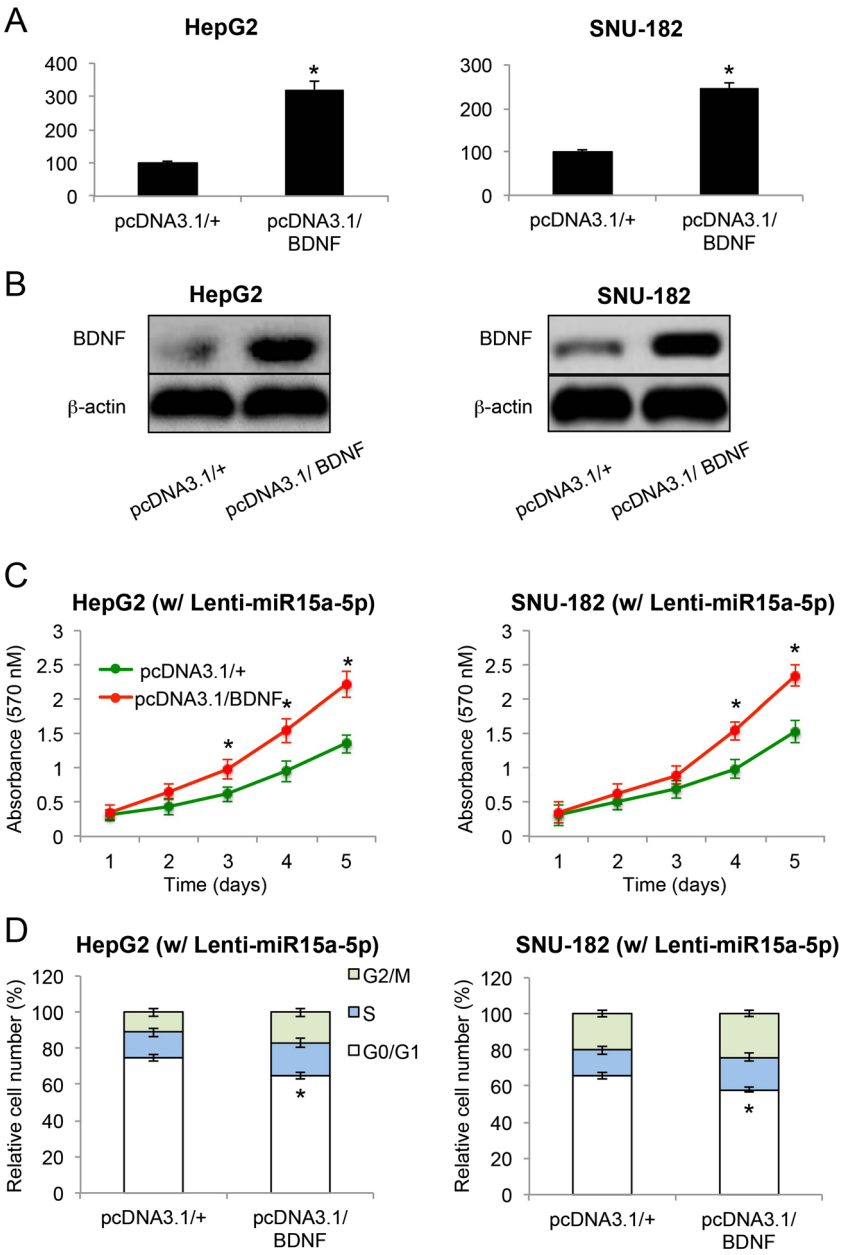

Fig. 5 BDNF overexpression overcame miR-15a-5p suppression on HCC proliferation and cell division. **a** HepG2 and SNU-182 cells were pre-transduced with Lenti-miR15a-5p, and then transfected with a BDNF overexpressing plasmid, pcDNA3.1/BDNF, or an empty plasmid pcDNA3.1. QRT-PCR was carried out to evaluate the gene expression of BDNF in miR-15a-5p overexpressed HepG2 and SNU-182 cells (*P<0.05). **b** After BDNF overexpression, Western blot was carried out to evaluate the protein expression of BDNF in miR-15a-5p overexpressed HepG2 and SNU-182 cells. **c** After BDNF overexpression, an in vitro MTT assay was carried out to evaluate cancer proliferation in HepG2 and SNU-182 cells pre-transduced with Lenti-miR15a-5p (*P<0.05). **d** After BDNF overexpression, a cell cycle assay was carried out to evaluate the percentages of HepG2 and SNU-182 cells in G0/G1, S, or G2/M stages (*P<0.05)

proliferation, cell division, metastasis, or apoptosis [17]. In this work, we demonstrated for the first time that miR-15a-5p was aberrantly downregulated in HCC cell lines, as compared to normal liver cell lines; and in HCC clinical specimens, as compared to adjacent non-tumor liver tissues. Previous studies showed that miR-15a-5p, mostly in miR-15a/miR-16 cluster, was downregulated in chronic lymphocytic leukemia [18], non-small cell lung cancer [19], and prostate cancer [12]. Thus, our finding suggested that miR-15a-5p had similar downregulated expression profile in HCC as in other cancer forms.

Functional experiments in our work showed that miR-15a-5p overexpression had tumor suppressive effects on HCC proliferation and cell division regulation. In chronic lymphocytic leukemia, the miR-15a/miR-16 cluster induced cancer cell apoptosis [18]. In prostate cancer, the miR-15a/miR-16 cluster was also shown to inhibit cancer survival, growth, and invasion [12]. These findings, along with ours in HCC, are consistent with the aberrant downregulation of miR-15a and support the tumor suppressor role of miR-15a in various cancer forms. Interestingly, our study is the first one to identify that miR-15a-5p itself had significant anti-tumor effects in HCC. Further experiments are needed to explore whether miR-16 also has the similar tumor suppressive mechanisms in HCC, as in other cancers. It would be also interesting to re-examine other cancers to see whether miR-15a-5p, among the miR-15a/miR-16 cluster, is the predominant factor to inhibit growth.

Also in our work, we identified that BDNF was the target gene of miR-15a-5p in regulating HCC. In other caners, miR-15a was found to be most likely targeting apoptosis or cell division related genes, such as BCL2 or CCND1, to induce cancer cell apoptosis or cell cycle arrest [12, 18, 20]. Thus, our report is the first one to reveal the association between miR-15a and BDNF in regulating cancer development. In various cancer forms, BDNF can be either oncogene or tumor suppressor. In melanoma and colon cancer, as well as breast cancer, BDNF is a tumor suppressor as activation or gene transfer of BDNF inhibited cancer proliferation [14, 21]. However, in human HCC, BDNF may act as an oncogene as BDNF was found to be upregulated in HCC and inhibition of BDNF induced apoptosis and suppressed invasion of HCC cell line HepG2 and HCCLM3 cells [16]. Therefore, the results in our work showing ectopic overexpression of BDNF ameliorated the inhibitory effect of miR-15a-5p on HCC proliferation and cell division further support the oncogenic role of BDNF in HCC.

Overall in this work, we showed for the first time that miR-15a-5p was aberrantly downregulated in both HCC cell lines and clinical HCC specimens. MiR-15a-5p is acting as a tumor suppressor in HCC as overexpression of miR-15a-5p reduced proliferation and induced cell cycle arrest in vitro, and inhibited tumor explants in vivo. We also elucidated the molecular pathways of miR-15a-5p in HCC, and demonstrated that BDNF was the target gene of miR-15a-5p, as well as an important regulator in miR-15a-5p-mediated HCC regulation. Those data may help to identify novel molecular pathways to provide targeted gene therapy for patients with HCC.

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Compliance with ethical standards

Conflicts of interest None

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